



PATENT

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THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:

ARTHUR SKOULTCHI

SPECIAL STATUS GRANTED

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For: PRODUCTION OF PROTEINS
USING HOMOLOGOUS
RECOMBINATION

Atty Docket No.: 7639-017/
Cell 3.2

DECLARATION OF MITCHELL FINER
UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, MITCHELL FINER, do declare that:

1. I am Director of the Molecular Biology Division of Cell Genesys, Inc., the assignee of the above-identified application. I have extensive experience in the field of molecular biology, particularly as it relates to gene expression and gene transfer, as evidenced by my curriculum vitae, attached hereto as Exhibit A.

2. The following analyses were carried out by me personally, or under my direction and supervision, to

EXPRESS MAIL CERTIFICATION

"Express Mail" Label No. EM 325 962 419US Date of Deposit October 25, 1995
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

FRANK DIANGELIS

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PENY3-420309.1

determine whether or not the erythropoietin (Epo) gene locus is normally transcriptionally silent in primary human 293 embryonal kidney cells (ATCC CRL 1573), the 293 cells used in the example at p.17 of the above-captioned application. The 293 cells used in the experiments described herein were obtained from the American Type Culture Collection (ATCC).

3. To test whether 293 cells contain the Epo gene, I obtained and analyzed from 293 cells, genomic DNA and total RNA, using the polymerase chain reaction (PCR) assays described below. PCR is a routinely used technique to selectively and repeatedly replicate target DNA sequences from a complex mixture of DNA, in order to detect a gene of interest in DNA and mRNA transcripts. Detection of Epo in genomic DNA indicates that the Epo gene is present in 293 cells. Detection of Epo mRNA indicates that 293 cells actively transcribe the Epo gene.

4. The following primers specific to regions across the Epo coding sequence (Genebank X02158; Jacobs et al., Nature 313, 806-810 (1985); Exhibit B) were designed and synthesized by Cell Genesys, and used as primers in the PCR reactions described below:

primer # 5000 5'-CTCACCAACATTGCTTGTGC-3';

primer # 5001 5'-TGCCACCTAAGTTCTCCAGG-3';

primer # 5002 5'-ACATTCCACAGAACTCACGC-3';

primer # 5004 5'-AGCAGGCCGTAGAAGTCT-3'; and

primer # 5005 5'-CTTTATCCACATGCAGCTGC-3'.

The regions of the Epo gene amplified by each pair is designated in Figure 1 (Exhibit C).

5. The β -actin gene is known to be actively transcribed in 293 cells, and therefore was analyzed as a positive control. The following PCR primers specific to human β -actin were synthesized by Cell Genyses, and used as positive controls in the PCR reactions described below:

Hu Beta Actin Upper 5' -CGAGCATCCCCCAAAGTTCACAA-3'; and

Hu Beta Actin Lower 5'-CCCAGCCACACCACAAAGTCACA-3'.

6. Genomic DNA was isolated from transformed primary human 293 embryonal kidney cells (ATCC CRL 1573). 100ng, 10ng, and 1ng of the 293 genomic DNA was analyzed by PCR using the following three pairs of primers specific for Epo: 5002/5001, 5000/5001, and 5004/5005.

7. The PCR analysis of genomic DNA isolated from 293 cells was carried out as follows: DNA samples in a volume (10 μ l) of TE were boiled for 10 minutes prior to the addition of the 1 x Taq polymerase reaction mix (40 μ l). The final reaction (50 μ l) contained 10 mM Tris-HCl (pH 9.0 at 25° C), 50 mM KCl, 1.5 mM MgCl₂, 100 μ M dNTPs, 50 pM of each primer, 1.0 U of Taq DNA polymerase (Promega) and an equal volume of Taq antibody (Promega). Following an initial incubation of 94°C for 3 minutes, the samples were subjected to 40 cycles of denaturation at 94°C for 30 sec., annealing at 60°C for 30 sec., and extension at 72°C for 30 sec. At the end of 40 cycles, a portion of each sample was analyzed on a 1% agarose

gel and stained with ethidium bromide as shown in Figure 2 (Exhibit D). These Epo primer pairs were able to reproducibly amplify Epo sequences throughout the gene. Thus, Figure 2 clearly demonstrates that the Epo gene is indeed present in the genome of 293 cells.

8. To determine whether the Epo gene is transcribed in 293 cells, total RNA obtained from the 293 cells was analyzed by reverse transcriptase-PCR (RT-PCR). RT will generate a cDNA copy of any mRNA transcripts present in the cellular RNA. Detection of a cDNA copy of Epo mRNA by PCR analysis indicates that the Epo gene is being transcribed.

9. RT reactions were carried out on 100ng of total RNA isolated from transformed primary human 293 embryonal kidney cells (ATCC CRL 1573). Total RNA isolated from human fetal liver was used as a positive Epo control. A random oligonucleotide hexamer (pd(N)₆) was used as the primer for the RT cDNA synthesis. RT reactions (25 μ l) contained 1x First Strand Synthesis buffer (Gibco-BRL), 1mM DTT, 3mM MgCl₂, 1.5 mM dNTPs, 25 pM/ μ l pd(N)₆, 2 U/ μ l Superscript RT (Gibco-BRL), 0.1 U/ μ l RNasin (Gibco-BRL) and 100ng of total RNA. Reaction samples were first incubated at 25°C for 10 min., followed by 37°C for 1 hour, and then heated to 95°C for 10 min. To control for DNA contamination in the RNA preparations, the RT enzyme was omitted from identical reactions.

10. PCR analysis was then carried out to determine the presence or absence of a cDNA copy of Epo mRNA.

Approximately 2 μ l of the 25 μ l RT reactions were analyzed by PCR. Epo PCR primer pair 5000/5001 and β -actin primers were used to amplify fragments from the cDNA. In every experiment primers specific to human β -actin were used as a control for integrity of output RNA and efficient RT reactions. The PCR reaction (50 μ l) contained 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 100 μ M dNTPs, 50 pM of each primer, 1.0 U of Taq DNA polymerase (Promega) and an equal volume of Taq antibody (Promega). Following an initial incubation of 94°C for 3 minutes, the samples were subjected to 40 cycles of denaturation at 94°C for 30 sec., annealing at 60°C for 30 sec., and extension at 72°C for 30 sec. At the end of 40 cycles, a portion of each sample was analyzed on a 1% agarose gel and stained with ethidium bromide (Figure 3; Exhibit E). A distinctive Epo band was detected in the RT+ lane of human fetal liver cDNA, indicating the presence of Epo mRNA in this control sample. No Epo bands were detected in either RT+ lane of the 293 total RNA, indicating the absence of Epo mRNA in this sample. β -actin fragments were also successfully amplified using total RNA from untargeted, naive 293 RT-PCR reactions, demonstrating the integrity of the total RNA from these cells.

11. The data described in paragraph 10 demonstrates that no cDNA copy of Epo mRNA was detected in the 293 cell samples, whereas a cDNA copy of β -actin mRNA was successfully detected indicating the integrity of RNA from these cells. On the basis of the foregoing data, I am able to conclude that

the Epo gene is normally transcriptionally silent in a primary human 293 embryonal kidney cell.

12. I declare further that all statements made in this Declaration of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: _____

10/20/95



Mitchell Finer